***Pappa2* deletion alters IGFBPs but has little effect on glucose disposal or adiposity**

Julian K. Christians, Amrit K. Bath and Neilab Amiri

Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, BC, V5A 1S6, Canada

Corresponding author:

Julian Christians

Department of Biological Sciences

Simon Fraser University

8888 University Drive, Burnaby, BC V5A 1S6

Phone: 778-782-5619

e-mail: jkchrist@sfu.ca

Short title: Effects of *Pappa2* deletion on metabolism

Abstract

Objective

Insulin-like growth factor binding proteins (IGFBPs) are involved in glucose and lipid metabolism, and their actions are modulated by proteases. The aim of this study was to examine the effects of an IGFBP-5 protease, pregnancy associated plasma protein -A2 (PAPP-A2), on glucose metabolism and susceptibility to diet-induced obesity.

Design

Postnatal growth, circulating IGF-I, IGFBP-3 and IGFBP-5 levels, and glucose tolerance were measured in *Pappa2* deletion mice and littermate controls on a chow diet. Males were subsequently fed a high-fat diet for 8 weeks to measure weight gain and adiposity, as well as glucose tolerance in response to a metabolic challenge.

Results

Circulating IGFBP-5 levels were ~2-fold higher in mice with no functional PAPP-A2 than in littermate controls, as expected. In contrast, circulating IGFBP-3 levels were reduced by ~15-fold, and total IGF-I levels were ~60% higher in *Pappa2* deletion mice. There was no effect of *Pappa2* deletion on fasting blood glucose levels or glucose clearance after intraperitoneal injection of 2 g glucose/kg body weight in mice on a chow diet. In males on a high-fat diet, there was no difference between genotypes in weight gain or adiposity, adjusting for differences in initial body weight, or in fasting blood glucose or insulin levels, or in glucose clearance.

Conclusions

Despite a dramatic disruption of the balance between circulating IGF-I, IGFBP-3 and -5, we found no effects of *Pappa2* deletion on glucose metabolism, weight gain or adiposity on a high-fat diet.

Keywords: Pappalysin-2; Insulin-like growth factor; Insulin-like growth factor binding protein; IGF-axis; glucose metabolism; adiposity

Abbreviations

ALS: acid-labile subunit

AUC: area under the curve

GTT: glucose tolerance test

HFD: high-fat diet

IGF: insulin-like growth factor

IGFBP: insulin-like growth factor binding protein

PAPP-A2: pregnancy associated plasma protein -A2

piAUC: positive incremental area under the curve

Introduction

Insulin-like growth factors (IGFs) and their binding proteins (IGFBPs) play roles in diverse processes including glucose metabolism and the regulation of adiposity [1-3]. IGFBPs not only sequester IGFs, reducing their availability, but also extend their half-life and in some cases have IGF-independent effects [1, 2, 4]. As a result, the various IGFBPs are not interchangeable. For example, overexpression of IGFBP-1 causes hyperglycemia and impairs glucose tolerance in mice [5] whereas overexpression of IGFBP-2 can reverse diabetes in mice [6], although this effect may be pharmacological [7].

IGFBPs are regulated by proteolysis [8]. Pregnancy associated plasma protein -A2 (PAPP-A2) is a protease of IGFBP-5 [9] that has been studied in the contexts of pregnancy [10-14] and postnatal growth [15-17]. Whether PAPP-A2 plays a role in the regulation of glucose metabolism and adiposity is unknown, but recently PAPP-A2 was found to be secreted in response to glucose in a model of murine pancreatic beta cells [18]. Furthermore, placental expression of PAPP-A2 is greatly downregulated in a mouse model of diabetic pregnancy [19]. PAPP-A2 is expected to influence IGFBP-5 levels through proteolysis, but might also influence IGFBP-3 levels indirectly, since there appears to be a link between circulating levels IGFBP-5 and IGFBP-3. Serum IGFBP-3 is elevated in *Igfbp5* null mice [20], and serum IGFBP-5 is elevated in *Igfbp3* null mice [21], whereas overexpression of *Igfbp5* reduces serum IGFBP-3 [22, 23]. It has been suggested that the inverse relationship between IGFBP-3 and -5 is not due to compensatory expression, but rather that elevated levels of one IGFBP leads to sequestration of IGF-I and/or acid-labile subunit (ALS) from the other IGFBP, rendering it susceptible to accelerated degradation [21, 24]. This competitive relationship would be particularly acute for IGFBP-3 and -5 since they are the only IGFBPs which bind to ALS [25].

Numerous transgenic mouse studies have investigated the effects of IGFBP-3 and -5 on glucose metabolism and adiposity. Deletion of *Igfbp5* results in hyperglycemia, slightly reduced glucose clearance in a glucose tolerance test (GTT), and increased adiposity on a high-fat diet [26]. Deletion of *Igfbp3* has no effect on fasting glucose levels and glucose clearance when mice are fed normal chow [21], but on a high-fat diet, *Igfbp3* null mice are hyperglycemic with normal glucose clearance and have less white adipose tissue than controls [27]. Triple knock-out mice with deletion of *Igfbp3, Igfbp4,* and *Igfbp5* have enhanced glucose clearance in GTT, reduced fasting blood glucose and reduced fat pad weight [28]. In contrast, *Igfbp3* overexpression leads to fasting hyperglycemia, impaired glucose clearance in GTT and, at least in one line, increased adiposity [29-31]. Because deletion of *Pappa2* would be expected to increase IGFBP-5 levels, but potentially reduce IGFBP-3 levels indirectly, its role in metabolism is impossible to predict. To our knowledge, no study has examined the effects of *Igfbp5* overexpression on glucose metabolism or adiposity.

The goal of this study was to examine whether deletion of *Pappa2* affected the circulating levels of IGFBP-5 and -3 and, if so, whether the change in the balance of IGFBPs affected glucose metabolism and susceptibility to diet-induced obesity. In addition to measuring glucose tolerance on standard chow, we also measured glucose tolerance and adiposity on a high-fat diet to determine whether there were greater differences between genotypes in response to a metabolic challenge [26, 27]. Since constitutive *Pappa2* deletion mice are smaller than wild-type [16], we also used a mouse model in which *Pappa2* was inactivated in adulthood to examine the relationship between circulating IGFBP-3 and -5, independent of body size.

Materials and methods

Pappa2 deletion mice

All work was carried out in accordance with the guidelines of the Canadian Council on Animal Care and approved by the SFU University Animal Care Committee (protocol 1035B-11). Mice were group-housed in individually ventilated cages (50 air changes/hour; max. 5 mice per cage) on a 12:12 hour light:dark cycle, at constant temperature (21 ± 1 °C), 50% humidity, with water and food (described below) available *ad libitum*. Conditional PAPP-A2 deletion mice with a C57BL/6 background were generated as previously described [16], such that mouse exon 2 (homologous to human exon 3) and a PGK-Neo selection cassette were flanked by LoxP sites (i.e., "floxed"). Since this previous work, the selection cassette was removed by FLP-FRT recombination to produce a floxed allele with no selection cassette (*Pappa2*^{fl}) and the *Flp* transgene was removed by further breeding. Furthermore, in mice carrying the constitutive *Pappa2* deletion allele (*Pappa2*^{KO}), the *Cre* transgene was removed by additional breeding . We have previously shown that PAPP-A2 protein is undetectable in placentae homozygous for the deletion allele, despite being abundant in wild-type mice [16].

In the present study, the conditional allele was used as the control for the deletion allele; there is no difference in postnatal weight gain between mice homozygous for the *Pappa2*^{fl} allele and littermates homozygous for the wild-type allele (described below). Mice heterozygous for the conditional and constitutive *Pappa2* alleles (*Pappa2*$^{fl/KO}$) were paired to produce litters in which all three genotypes were present (i.e., *Pappa2*$^{fl/fl}$, *Pappa2*$^{fl/KO}$, *Pappa2*$^{KO/KO}$). Mice were weaned at approximately three weeks of age and maintained on breeding chow (Prolab RMH 2000 5P06, 23% kcal from fat, LabDiet, St. Louis, MO) until 6 weeks of age, when they were switched to normal chow (5001, 13.5% kcal from fat, LabDiet, St. Louis, MO). Mice were ear-clipped at weaning and extraction of DNA and PCR genotyping were performed by standard

methods. Primer sequences are as follows: KO_prox (5'-CAGCAAAGGAAATTTGTGCT-3'), KO_exon2 (5'-GGTCAAATGAAACTTCCCTCC-3'), KO_dist2 (5'-CTCTTGCATGCCTCCACTAC-3').

Male and female mice were blood sampled from the saphenous vein at 6 weeks of age for measurement of circulating IGF-I, IGFBP-3 and IGFBP-5 levels (N= 12-17 per genotype), and glucose tolerance testing was performed at 11 weeks of age (N= 17-29 per genotype). Body weight and tail length were measured at 3, 6, 10 and 14 weeks of age (N= 32-57 per genotype). Mice were fed a high-fat diet (45% kcal from fat, D12451, Research Diets, New Brunswick, NJ) from 17 weeks of age until 25 weeks of age, when glucose tolerance tests were performed and mice were culled (N= 10-11 per genotype). The high-fat diet experiment used only male heterozygotes and homozygous deletion mice (i.e., $Pappa2^{fl/KO}$ and $Pappa2^{KO/KO}$) since females and male homozygous for the floxed allele were used for other experiments. In previous studies, the effects of *Pappa2* deletion on postnatal growth have been found to be completely recessive [16], and in the present study we also found effects on circulating IGF and IGFBPs to be mostly recessive (described below). In almost all cases in the high-fat diet experiment, $Pappa2^{KO/KO}$ males were matched with $Pappa2^{fl/KO}$ littermates, with siblings kept in the same cage throughout the experiment, precluding the collection of individual food consumption data.

Adult-specific Pappa2 *deletion mice*

Adult-specific *Pappa2* deletion was achieved by crossing conditional deletion mice ($Pappa2^{fl/fl}$) to mice with tamoxifen-inducible *Cre* recombinase expression (hereafter referred to as *Cre-ERT2*; Jackson Laboratory stock number 008085). Resulting offspring carrying the transgene ($Pappa2^{wt/fl}$; *Cre-ERT2*) were mated to mice heterozygous for the conditional allele ($Pappa2^{wt/fl}$)

and the body weight and tail length of offspring were measured at 3, 6, 10, 14 and 18 weeks of age (N= 10-22 per genotype, with 6 genotypes: three *Pappa2* genotypes, each with or without the *Cre-ERT2* transgene). *Pappa2* genotype was determined as described above, while *Cre-ERT2* transgene genotype was determined using two primer pairs recommended by the Jackson Laboratory: one to amplify a fragment of the transgene and another to amplify a positive control fragment to confirm that the PCR was successful. Primer sequences are as follows: Cre_A (5'-GCGGTCTGGCAGTAAAAACTATC-3'), Cre_B (5'-GTGAAACAGCATTGCTGTCACTT-3'), Cre_+ve_A (5'-CTAGGCCACAGAATTGAAAGATCT-3'), Cre_+ve_B (5'-GTAGGTGGAAATTCTAGCATCATCC-3').

Offspring homozygous for the wild-type allele or the floxed allele and that carried the transgene (*Pappa2*$^{wt/wt}$; *Cre-ERT2* or *Pappa2*$^{fl/fl}$; *Cre-ERT2*) were treated with tamoxifen (Sigma-Aldrich) in corn oil at a dose of 75 mg / kg body weight by intraperitoneal injection once per day for 5 consecutive days, as validated by the Jackson Laboratory for this *Cre* line. Mice were between 18 and 27 weeks of age (median age: 20 weeks) at the time of the first tamoxifen injection, and were blood sampled 7 weeks later (N= 11-14 per genotype).

Glucose tolerance tests and measurement of fat depots

Glucose tolerance tests were performed after a 5 hour fast on unanesthetized animals [32, 33]. Mice were given an intraperitoneal injection of 20% glucose at a dose of 2 g D-glucose/kg body weight [34], and blood sampled from the saphenous vein at 0, 15, 30, 60 and 120 minutes after injection. Blood glucose levels were measured using an AlphaTRAK 2 glucometer (Abbott, Illinois). A plasma sample taken immediately prior to glucose challenge was frozen for measurement of insulin. All glucose tolerance tests were performed at the same time of day. For

mice on a high-fat diet, the glucose dose was based on body weight at the initiation of the high-fat diet as a proxy for lean body weight, to reduce variation in the glucose dose per weight of lean tissue [32].

Mice on a high-fat diet were sacrificed the day following the glucose tolerance test and frozen. Mice were later thawed and the gonadal, retroperitoneal, mesenteric, and omental fat depots were removed, and dried to a constant weight. These four depots were selected since visceral fat is associated with risk of diabetes [35].

Enzyme linked immunosorbent assays (ELISA)

Plasma levels of IGF-I, IGFBP-3, IGFBP-5 were measured in triplicate by species-specific ELISA (MG100, MGB300, and DY578, respectively, R&D Systems, Minneapolis, MN) at dilutions of 1:500, 1:300 and 1:75, respectively. Plasma insulin was measured in triplicate by species-specific ELISA (90080, Crystal Chem, Downers Grove, IL) in undiluted samples. According to the manufacturer, these ELISAs show no significant cross-reactivity with related mouse proteins (including other IGFBPs).

Quantitative PCR

We measured *Igfbp3* and *Igfbp5* transcript levels in 8 *Pappa2*$^{fl/fl}$ (4 females and 4 males) and 7 *Pappa2*$^{KO/KO}$ (3 females and 4 males) mice. Males were collected between 20 and 27 weeks of age and females were collected between 24 and 32 weeks of age. A sample of kidney and liver were dissected immediately after sacrifice and stored in RNAlater (Ambion, Foster City, CA). We measured mRNA levels in kidney and in liver because *Igfbp3* expression is particularly strong in these tissues [36, 37]. Tissue was homogenized at room temperature in 600 μL of

buffer RLT (Qiagen, Ontario, Canada) using pestles and Qiashredders (Qiagen, Ontario, Canada) and total RNA was extracted using the RNeasy Mini kit (Qiagen, Ontario, Canada). RNA concentration was determined using a Nanodrop spectrophotometer (Thermo Fischer Scientific Inc. Waltham, MA), and each sample was diluted to a final concentration of 50 ng/μL. A reference sample was prepared by combining samples and was included in every assay to account for variation between assays. Levels of *β-actin* were also measured as a reference. Primer sequences were obtained from [23] and are as follows: *Igfbp3*: 5'-CCAGAACTTCTCCTCCGAGTCTAAG-3' and 5'-CTCAGCACATTGAGGAACTTCAGAT-3'; *Igfbp5*: 5'-AGATGGCTGAAGAGACCTACTCC-3' and 5'-GCTTTCTCTTGTAGAATCCTTTG-3'; *β-actin*: 5'-CAGGTCATCACTATTGGCAACGAG-3' and 5'-ACGGATGTCAACGTCACACTTCAT-3'. The qScript 1-step SYBR Green qRT-PCR kit (Quanta Biosciences Inc. Gaithersburg, MD) was used to reverse-transcribe and amplify each sample for 40 cycles. At each cycle, the amount of fluorescence was quantified using a miniOpticon (Bio-Rad, Hercules, CA), and the cycle at which the signal rose above a fixed threshold (Ct) was determined. Each sample was analysed in duplicate. We used the method of Pfaffl [38] to calculate mRNA expression levels for *Igfbp3* and *Igfbp5*, relative to the reference sample, e.g., a value of 1.5 indicates a sample has 50% more of a particular transcript than the reference sample, correcting for *β-actin*.

Statistical analyses

All statistical analyses were performed using general linear models (proc GLM) or repeated measures analyses (proc MIXED) in SAS, Version 9.3 (SAS Institute Inc., Cary, NC). Terms included in the models are described in the text, figure legends and footnotes to the tables.

Results

Postnatal growth

Mice homozygous for the *Pappa2* deletion were lighter than littermates heterozygous or homozygous for the floxed (i.e., intact) allele at 3, 6, 10 and 14 weeks of age (Fig. 1A, Supplementary Table 1), as previously observed comparing homozygous deletion mice with wild-type mice [16]. As in previous work comparing deletion and wild-type mice, the phenotype of heterozygotes was closer to that of homozygous floxed mice (Supplementary Table 1) and homozygous deletion mice were significantly smaller within each sex (data not shown).

Circulating levels of IGFBP-5, IGFBP-3 and IGF-I

As expected, circulating IGFBP-5 levels at 6 weeks of age were significantly higher in mice with no functional PAPP-A2 than in littermates with one or two floxed alleles (Table 1). While the mean IGFBP-5 level in heterozygotes was significantly different from both $Pappa2^{KO/KO}$ and $Pappa2^{fl/fl}$ mice, it was closer to $Pappa2^{fl/fl}$ levels. In contrast, circulating IGFBP-3 levels were dramatically lower in $Pappa2^{KO/KO}$ mice, with heterozygotes significantly different from both $Pappa2^{KO/KO}$ and $Pappa2^{fl/fl}$ mice, but much closer to the latter (Table 1).

Total IGF-I levels were significantly higher in $Pappa2^{KO/KO}$ mice, with levels in heterozygotes not significantly different from $Pappa2^{fl/fl}$ levels (Table 1). For circulating IGF-I or IGFBP-3 levels, the genotype by sex interaction was not significant, and there was no difference between the sexes (data not shown). In contrast, the genotype by sex interaction was significant for IGFBP-5 levels ($F_{2,28} = 3.42$, $P = 0.047$), although in each sex analysed separately, circulating IGFBP-5 was significantly higher in homozygous deletion mice (data not shown).

There was also a significant effect of sex, with circulating IGFBP-5 levels significantly higher in females ($F_{1,28}$ = 12.84, P = 0.0013; least squares means: males: 131 ± 3 ng/mL; females: 147 ± 3 ng/mL).

Igfbp3 *and* Igfbp5 *expression in liver and kidney*

We tested whether *Igfbp3* expression was altered in the liver or kidney of *Pappa2* deletion mice. At cull between 21 and 32 weeks of age (median age: 27 weeks), circulating IGFBP-5 and IGFBP-3 were still higher and lower, respectively, in *Pappa2* deletion mice, as observed at 6 weeks of age (Table 1). However, there was no effect of *Pappa2* deletion on *Igfbp3* or *Igfbp5* expression at the mRNA level in either the kidney or the liver (Table 1). The genotype by sex interaction was not significant for any of the traits measured at cull (P > 0.05), and there was only a significant effect of sex for circulating IGFBP-5 levels and liver *Igfbp5* mRNA. As at 6 weeks of age, circulating IGFBP-5 levels at cull were significantly higher in females ($F_{1,12}$ = 13.87, P = 0.0029; males: 92 ± 6 ng/mL; females: 127 ± 7 ng/mL), as was liver *Igfbp5* mRNA ($F_{1,12}$ = 17.94, P = 0.0012; males: 1.1 ± 0.3; females: 3.1 ± 0.4; mRNA units are fold-difference compared to a reference sample and corrected for *β-actin*).

Adult-specific Pappa2 *deletion*

To investigate the effects of *Pappa2* deletion on circulating IGFBP-3 and -5, independent of body size, we generated mice in which *Pappa2* was inactivated during adulthood by tamoxifen-induced *Cre*-mediated recombination. Prior to tamoxifen administration, there was no effect of *Pappa2* genotype or the presence of the *Cre* transgene on body weight at 3, 6, 10, 14 or 18 weeks of age (Fig. 1B; Supplementary Table 2). The lack of phenotypic difference between *Pappa2*$^{fl/fl}$

and *Pappa2*$^{wt/wt}$ littermates supports the use of *Pappa2*$^{fl/fl}$ mice as controls for *Pappa2*$^{KO/KO}$ mice. Seven weeks after the first tamoxifen injection, there was no difference in body weight between *Pappa2*$^{fl/fl}$ and *Pappa2*$^{wt/wt}$ mice carrying the tamoxifen-inducible *Cre* transgene ($F_{1,14} = 0.80$; $P = 0.39$; Table 2). Circulating IGFBP-5 levels did not differ between genotypes ($F_{1,14} = 2.69$; $P = 0.12$; Table 2), although the average value was higher in adult-specific deletion mice (*Pappa2*$^{fl/fl}$) than in tamoxifen-treated wild-type mice (*Pappa2*$^{wt/wt}$), as expected. As in constitutive deletion mice, circulating IGFBP-5 levels were significantly higher in females than in males ($F_{1,14} = 22.92$; $P = 0.0003$; males: 82 ± 16 ng/mL; females: 200 ± 15 ng/mL). Despite the lack of an effect on IGFBP-5 levels, circulating IGFBP-3 levels were significantly lower in adult-specific deletion mice than in tamoxifen-treated controls ($F_{1,14} = 13.41$; $P = 0.0026$; Table 2), indicating that the effect of *Pappa2* deletion on circulating IGFBP-3 is independent of body size. However, the reduction in IGFBP-3 levels in adult-specific deletion mice was much smaller than that in the constitutive deletion mice, potentially because of incomplete inactivation of *Pappa2* (Fig. 2). Circulating IGFBP-3 levels did not differ between the sexes ($F_{1,14} = 0.02$; $P = 0.90$).

Glucose tolerance tests on chow

We analysed blood glucose levels using a general linear model including effects of *Pappa2* genotype, sex, genotype by sex interaction, and batch (i.e., the day on which the glucose tolerance test was performed, to account for variation between test days). The genotype by sex interaction term was not significant at any time point and so was removed from the model, i.e., there was no evidence of sex-specific effects of *Pappa2* genotype.

On a chow diet, baseline blood glucose levels did not differ among *Pappa2* genotypes ($F_{2,58} = 0.09$; $P = 0.92$; Table 3), and were significantly higher in males than females ($F_{1,58} =$

6.61; P = 0.01; males: 9.8 ± 0.2 mmol/L; females: 9.0 ± 0.2 mmol/L). Glucose levels did not differ between *Pappa2* genotypes at any point after glucose injection (Fig. 3A). However, the variation among genotypes was marginally non-significant at 15 minutes after injection ($F_{2,55}$ = 3.05; P = 0.06) (Fig. 3A). There was no difference among *Pappa2* genotypes in the area under the curve (AUC) ($F_{2,54}$ = 0.76; P = 0.47; Table 3), or the positive incremental area under the curve (i.e., the area under the curve, but above the baseline level, piAUC) ($F_{2,54}$ = 0.91; P = 0.41; Table 3). Males had a higher AUC than females ($F_{1,54}$ = 8.03; P = 0.006; males: 30.7 ± 0.5 mmol*hour/L; females: 28.5 ± 0.6 mmol*hour/L), but piAUC did not differ between the sexes ($F_{1,54}$ = 0.92; P = 0.34; males: 11.4 ± 0.5 mmol*hour/L; females: 10.7 ± 0.6 mmol*hour/L).

Glucose tolerance tests, weight gain and fat depots on high-fat diet

We fed male *Pappa2*$^{KO/KO}$ and *Pappa2*$^{fl/KO}$ mice a high-fat diet to determine whether there were greater differences between genotypes in response to a metabolic challenge. Weight gain and relative weight (gain expressed as percentage of initial weight) were analysed by repeated measures analysis using the MIXED procedure (SAS 9.3, SAS Institute) and individual mouse as the subject. The difference between genotypes was significant in the repeated measures analysis for both weight gain ($F_{1,19}$ = 77.11; P < 0.0001) and body weight as a percentage of initial weight ($F_{1,19}$ = 28.84; P < 0.0001). On a high-fat diet, *Pappa2*$^{KO/KO}$ mice gained significantly less weight than *Pappa2*$^{fl/KO}$ mice in absolute terms, and in terms of body weight as a percentage of weight at the initiation of the high-fat diet (Fig. 4A, B). However, there was no difference between genotypes in body weight after 8 weeks on the high-fat diet ($F_{1,18}$ = 1.37; P = 0.26), controlling for body weight at the initiation of the high-fat diet in a general linear model, i.e., the

relationship between initial and final weight followed the same pattern for both genotypes (Fig. 5A). This suggests that the difference in absolute and proportional weight gain was due to the lower starting weight of *Pappa2*$^{KO/KO}$ mice, and not to a difference in metabolism between genotypes *per se*.

We analysed differences in the weights of fat depots between genotypes in three ways: absolute weight, weight as a percentage of body weight, and controlling for body weight as a covariate in the model. The absolute weights of the gonadal and retroperitoneal fat depots and the total weight of the fat depots were significantly lower in *Pappa2*$^{KO/KO}$ mice, while the weight of the omental fat depot was marginally non-significantly lower in *Pappa2*$^{KO/KO}$ mice, and the weight of the mesenteric fat depot did not differ between genotypes (Table 4). As a percentage of body weight, the retroperitoneal fat depot was significantly smaller in *Pappa2*$^{KO/KO}$ mice, but there was no significant difference in the other depots (Table 4). As a percentage of body weight, total weight of the fat depots tended to be lower in *Pappa2*$^{KO/KO}$ mice, but this difference was marginally non-significant (P = 0.07) (Table 4). However, controlling for body weight as a covariate in the model, the only significant difference between genotypes was in the weight of the mesenteric fat depot, which was higher in *Pappa2*$^{KO/KO}$ mice. As with gains in body weight, the relationship between total fat weight and body weight followed a similar pattern for both genotypes (Fig. 5B), suggesting that the difference in body fat was due to the lower weight of *Pappa2*$^{KO/KO}$ mice, and not to a difference in metabolism between genotypes.

On a high-fat diet, there was no difference among *Pappa2* genotypes in fasting baseline blood glucose ($F_{1,19}$ = 0.18; P = 0.67; Table 3) or insulin levels ($F_{1,18}$ = 0.00; P = 0.99; Table 3). As expected, older (25 week old) mice on a high-fat diet had an impaired response to the glucose challenge compared with younger (11 week old) mice on chow, with a higher peak in blood

glucose and higher blood glucose two hours after injection (Fig. 3). The glucose dose was based on their weight at the initiation of the high-fat diet, and therefore these mice actually received a lower dose of glucose per actual weight compared with the mice on chow. Glucose levels did not differ between *Pappa2* genotypes at any point after glucose injection (Fig. 3B), and there was no difference in AUC or piAUC between *Pappa2* genotypes ($P > 0.5$ in both cases; Table 3).

Discussion

IGFBP proteases add another layer of complexity to the roles of the IGFs and IGFBPs in metabolism since they not only decrease the levels of their target(s), but may indirectly increase the levels of other IGFBPs and/or trigger effects of IGFBP proteolytic fragments. We investigated the effects of deleting an IGFBP-5 protease, *Pappa2*, and found that circulating IGFBP-5 levels were increased, as expected. Because circulating IGFBP-5 competes for ALS with IGFBP-3 but not other IGFBPs [25], we also measured circulating IGFBP-3. Deletion of *Pappa2* dramatically reduced levels of IGFBP-3, usually the predominant IGFBP in circulation [4], consistent with previous studies of *Igfbp5* deletion or overexpression [20, 22, 23]. Furthermore, IGFBP-3 was also reduced by adult-specific deletion of *Pappa2*, indicating that this effect was not due to compensation for reduced body size. The decrease in circulating IGFBP-3 was not accompanied by decreased levels of *Igfbp3* mRNA in the liver or kidney, consistent with the hypothesis that the reduction in IGFBP-3 was due to sequestration of IGF-I and/or ALS by excess IGFBP-5, rendering IGFBP-3 more susceptible to degradation.

While deletion of *Pappa2* would be expected to have effects similar to *Igfbp5* overexpression, these studies [22, 23] did not report effects on glucose metabolism or adiposity.

Since *Pappa2* deletion also reduced IGFBP-3 levels substantially, this manipulation might be similar to *Igfbp3* deletion. As with *Igfbp3* null mice on a chow diet [21], we found no effect of *Pappa2* deletion on fasting glucose levels or glucose clearance in a glucose tolerance test. On a high-fat diet, *Igfbp3* null mice had higher fasting blood glucose and insulin levels and lower epididymal fat pad weight than controls, while glucose clearance was unaffected by *Igfbp3* deletion [27]. In contrast, *Pappa2* deletion did not affect fasting blood glucose, insulin levels or adiposity on a high-fat diet. Deletion of *Pappa,* a paralog of *Pappa2* that encodes a protease of both IGFBP-4 and -5, had no effect on fasting glucose or insulin levels or glucose clearance [39]. In *Pappa* deletion mice on a high-fat diet, there were reductions in the weights of some fat depots, particularly in females, although these were analysed as percentage of body weight [40].

After 8 weeks on a high-fat diet, *Pappa2* deletion mice gained less weight than controls and had lighter fat depots in absolute terms. Similarly, as a proportion of body weight, *Pappa2* deletion mice also gained less weight than controls, and had a decreased total weight of fat depots, although this latter difference was marginally non-significant ($P = 0.07$). In contrast, when adjusting for body weight by including this term as a covariate rather than using proportions, there was no effect of genotype on weight gain or the total weight of fat stores. The discrepancy between our analyses using proportions and those adjusting for body weight as a covariate illustrates the pitfalls of using ratios to adjust for body weight. The use of ratios is only valid if the relationship between the trait of interest and body size is isometric, i.e., that it is linear and passes through the origin [41].

There is interest in IGFBPs as therapeutic targets in the treatment of diabetes and obesity, but our knowledge of the effects of individual components of the IGF system and their interactions is limited [42]. We deleted an IGFBP protease, and therefore manipulated relative

IGFBP levels without inducing pharmacological effects potentially observed in overexpression studies. Deletion of *Pappa2* in mice increased circulating IGFBP-5 levels, as expected, and also decreased circulating IGFBP-3. *Pappa2* deletion increased IGF-I levels, but decreased postnatal growth, as observed in *Igfbp5*-overexpressing mice [23]. In both of these studies, the reason for the increased IGF-I levels remains unknown

Despite the dramatic disruption of the balance between circulating IGF-I, IGFBP-3 and -5, we found no effects of *Pappa2* deletion on glucose metabolism, either on a standard chow diet or a high-fat diet. There were no effects of *Pappa2* deletion on weight gain on a high-fat diet, or the total weight of fat depots, when correcting for body size. Because we deleted *Pappa2* globally, we cannot rule out the possibility that the effects of PAPP-A2 on circulating IGFBP levels may have been counteracted by effects on local or circulating IGF-I bioavailability, IGF-independent pathways, or even IGFBP-5 independent pathways [43].

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References

[1] H. Kim, Role of insulin-like growth factor binding protein-3 in glucose and lipid metabolism. Annals of Pediatric Endocrinology and Metabolism 18 (1) (2013) 9-12.

[2] S.B. Wheatcroft, M.T. Kearney, IGF-dependent and IGF-independent actions of IGF-binding protein-1 and-2: Implications for metabolic homeostasis. Trends in Endocrinology and Metabolism 20 (4) (2009) 153-162.

[3] W. Ruan, M. Lai, Insulin-like growth factor binding protein: A possible marker for the metabolic syndrome? Acta Diabetol. 47 (1) (2010) 5-14.

[4] P.M. Yamada, K. Lee, Perspectives in mammalian IGFBP-3 biology: Local vs. systemic action. American Journal of Physiology-Cell Physiology 296 (5) (2009) C954-C976.

[5] K. Rajkumar, S.T. Dheen, L.J. Murphy, Hyperglycemia and impaired glucose tolerance in IGF binding protein-1 transgenic mice. American Journal of Physiology-Endocrinology and Metabolism 270 (4) (1996) E565-E571.

[6] K. Hedbacker, K. Birsoy, R.W. Wysocki, E. Asilmaz, R.S. Ahima, I.S. Farooqi, J.M. Friedman, Antidiabetic effects of IGFBP2, a leptin-regulated gene. Cell Metabolism 11 (1) (2010) 11-22.

[7] U.H. Neumann, S. Chen, Y.Y.C. Tam, R.K. Baker, S.D. Covey, P.R. Cullis, T.J. Kieffer, IGFBP2 is neither sufficient nor necessary for the physiological actions of leptin on glucose homeostasis in male ob/ob mice. Endocrinology 155 (3) (2014) 716-725.

[8] R.C. Bunn, J.L. Fowlkes, Insulin-like growth factor binding protein proteolysis. Trends in Endocrinology and Metabolism 14 (4) (2003) 176-181.

[9] M.T. Overgaard, H.B. Boldt, L.S. Laursen, L. Sottrup-Jensen, C.A. Conover, C. Oxvig, Pregnancy-associated plasma protein-A2 (PAPP-A2), a novel insulin-like growth factor-binding protein-5 proteinase. J. Biol. Chem. 276 (24) (2001) 21849-21853.

[10] X.L. Yan, R.C. Baxter, S.M. Firth, Involvement of pregnancy-associated plasma protein-A2 in insulin-like growth factor (IGF) binding protein-5 proteolysis during pregnancy: A potential mechanism for increasing IGF bioavailability. J Clin Endocrinol Metab 95 (3) (2010) 1412-1420.

[11] P.K. Wagner, J.K. Christians, Altered placental expression of PAPPA2 does not affect birth weight in mice. Reprod Biol Endocrin 8 (2010) 90.

[12] J. Wang, Q. Qiu, M. Haider, M. Bell, A. Gruslin, J.K. Christians, Expression of pregnancy-associated plasma protein A2 during pregnancy in human and mouse. J. Endocrinol. 202 (3) (2009) 337-345.

[13] E.J. Crosley, U. Durland, K. Seethram, S. Macrae, A. Gruslin, J.K. Christians, First-trimester levels of pregnancy-associated plasma protein A2 (PAPP-A2) in the maternal circulation are elevated in pregnancies that subsequently develop preeclampsia. Reprod. Sci. 21 (6) (2014) 754-760.

[14] P.K. Wagner, A. Otomo, J.K. Christians, Regulation of pregnancy-associated plasma protein A2 (PAPPA2) in a human placental trophoblast cell line (BeWo). Reproductive Biology and Endocrinology 9 (2011) 48.

[15] J.K. Christians, A. Hoeflich, P.D. Keightley, PAPPA2, an enzyme that cleaves an insulin-like growth-factor-binding protein, is a candidate gene for a quantitative trait locus affecting body size in mice. Genetics 173 (3) (2006) 1547-1553.

[16] J.K. Christians, D.R. de Zwaan, S.H.Y. Fung, Pregnancy associated plasma protein A2 (PAPP-A2) affects bone size and shape and contributes to natural variation in postnatal growth in mice. Plos One 8 (2) (2013) e56260.

[17] C.A. Conover, H.B. Boldt, L.K. Bale, K.B. Clifton, J.A. Grell, J.R. Mader, E.J. Mason, D.R. Powell, Pregnancy-associated plasma protein-A2 (PAPP-A2): Tissue expression and biological consequences of gene knockout in mice. Endocrinology 152 (7) (2011) 2837-2844.

[18] S.G. Tattikota, M.D. Sury, T. Rathjen, H. Wessels, A.K. Pandey, X. You, C. Becker, W. Chen, M. Selbach, M.N. Poy, Argonaute2 regulates the pancreatic beta-cell secretome. Molecular & Cellular Proteomics 12 (5) (2013) 1214-1225.

[19] C. Kappen, C. Kruger, J. MacGowan, J.M. Salbaum, Maternal diet modulates placenta growth and gene expression in a mouse model of diabetic pregnancy. Plos One 7 (6) (2012) e38445.

[20] Y. Ning, B. Hoang, A.G.P. Schuller, T.P. Cominski, M. Hsu, T.L. Wood, J.E. Pintar, Delayed mammary gland involution in mice with mutation of the insulin-like growth factor binding protein 5 gene. Endocrinology 148 (5) (2007) 2138-2147.

[21] S. Yakar, C.J. Rosen, M.L. Bouxsein, H. Sun, W. Mejia, Y. Kawashima, Y. Wu, K. Emerton, V. Williams, K. Jepsen, M.B. Schaffler, R.J. Majeska, O. Gavrilova, M. Gutierrez, D.

Hwang, P. Pennisi, J. Frystyk, Y. Boisclair, J. Pintar, H. Jasper, H. Domene, P. Cohen, D. Clemmons, D. LeRoith, Serum complexes of insulin-like growth factor-1 modulate skeletal integrity and carbohydrate metabolism. Faseb Journal 23 (3) (2009) 709-719.

[22] G. Tripathi, D.A.M. Salih, A.C. Drozd, R.A. Cosgrove, L.J. Cobb, J.M. Pell, IGF-independent effects of insulin-like growth factor binding protein-5 (Igfbp5) in vivo. Faseb Journal 23 (8) (2009) 2616-2626.

[23] D.A.M. Salih, G. Tripathi, C. Holding, T.A.M. Szestak, M.L. Gonzalez, E.J. Carter, L.J. Cobb, J.E. Eisemann, J.M. Pell, Insulin-like growth factor-binding protein 5 (Igfbp5) compromises survival, growth, muscle development, and fertility in mice. Proc. Natl. Acad. Sci. U. S. A. 101 (12) (2004) 4314-4319.

[24] I. Ueki, G.T. Ooi, M.L. Tremblay, K.R. Hurst, L.A. Bach, Y.R. Boisclair, Inactivation of the acid labile subunit gene in mice results in mild retardation of postnatal growth despite profound disruptions in the circulating insulin-like growth factor system. Proc. Natl. Acad. Sci. U. S. A. 97 (12) (2000) 6868-6873.

[25] S.M. Twigg, R.C. Baxter, Insulin-like growth factor (IGF)-binding protein 5 forms an alternative ternary complex with IGFs and the acid-labile subunit. J. Biol. Chem. 273 (11) (1998) 6074-6079.

[26] C.E. Gleason, Y. Ning, T.P. Cominski, R. Gupta, K.H. Kaestner, J.E. Pintar, M.J. Birnbaum, Role of insulin-like growth factor-binding protein 5 (IGFBP5) in organismal and pancreatic beta-cell growth. Molecular Endocrinology 24 (1) (2010) 178-192.

[27] P.M. Yamada, H.H. Mehta, D. Hwang, K.P. Roos, A.L. Hevener, K.W. Lee, Evidence of a role for insulin-like growth factor binding protein (IGFBP)-3 in metabolic regulation. Endocrinology 151 (12) (2010) 5741-5750.

[28] Y. Ning, A.G.P. Schuller, S. Bradshaw, P. Rotwein, T. Ludwig, J. Frystyk, J.E. Pintar, Diminished growth and enhanced glucose metabolism in triple knockout mice containing mutations of insulin-like growth factor binding protein-3,-4, and-5. Molecular Endocrinology 20 (9) (2006) 2173-2186.

[29] J.V. Silha, Y.T. Gui, L.J. Murphy, Impaired glucose homeostasis in insulin-like growth factor-binding protein-3-transgenic mice. American Journal of Physiology-Endocrinology and Metabolism 283 (5) (2002) E937-E945.

[30] K.H. Nguyen, X. Yao, S. Moulik, S. Mishra, B.L.G. Nyomba, Human IGF binding protein-3 overexpression impairs glucose regulation in mice via an inhibition of insulin secretion. Endocrinology 152 (6) (2011) 2184-2196.

[31] T. Modric, J.V. Silha, Z. Shi, Y. Gui, A. Suwanichkul, S.K. Durham, D.R. Powell, L.J. Murphy, Phenotypic manifestations of insulin-like growth factor-binding protein-3 overexpression in transgenic mice. Endocrinology 142 (5) (2001) 1958-1967.

[32] J.E. Ayala, V.T. Samuel, G.J. Morton, S. Obici, C.M. Croniger, G.I. Shulman, D.H. Wasserman, O.P. McGuinness, NIH Mouse Metab Phenotyping Ctr Co, Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. Disease Models & Mechanisms 3 (9-10) (2010) 525-534.

[33] J.E. Bowe, Z.J. Franklin, A.C. Hauge-Evans, A.J. King, S.J. Persaud, P.M. Jones, METABOLIC PHENOTYPING GUIDELINES assessing glucose homeostasis in rodent models. J. Endocrinol. 222 (3) (2014) G13-G25.

[34] S. Andrikopoulos, A.R. Blair, N. Deluca, B.C. Fam, J. Proietto, Evaluating the glucose tolerance test in mice. American Journal of Physiology-Endocrinology and Metabolism 295 (6) (2008) E1323-E1332.

[35] R.N. Bergman, S.P. Kim, K.J. Catalano, I.R. Hsu, J.D. Chiu, M. Kabir, K. Hucking, M. Ader, Why visceral fat is bad: Mechanisms of the metabolic syndrome. Obesity 14 (2006) 16S-19S.

[36] A.G.P. Schuller, C. Groffen, J.W. Vanneck, E.C. Zwarthoff, S.L.S. Drop, Cdna cloning and messenger-rna expression of the 6 mouse insulin-like growth-factor binding-proteins. Mol. Cell. Endocrinol. 104 (1) (1994) 57-66.

[37] A.L. Albiston, A.C. Herington, Tissue distribution and regulation of insulin-like growth-factor (igf)-binding protein-3 messenger-ribonucleic-acid (messenger-rna) in the rat - comparison with igf-i messenger-rna expression. Endocrinology 130 (1) (1992) 497-502.

[38] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29 (9) (2001) e45.

[39] C.A. Conover, M.A. Mason, J.A. Levine, C.M. Novak, Metabolic consequences of pregnancy-associated plasma protein-A deficiency in mice: Exploring possible relationship to the longevity phenotype. J. Endocrinol. 198 (3) (2008) 599-605.

[40] C.A. Conover, S.L. Harstad, T. Tchkonia, J.L. Kirkland, Preferential impact of pregnancy-associated plasma protein-A deficiency on visceral fat in mice on high-fat diet. American Journal of Physiology-Endocrinology and Metabolism 305 (9) (2013) E1145-E1153.

[41] G.C. Packard, T.J. Boardman, The use of percentages and size-specific indices to normalize physiological data for variation in body size: Wasted time, wasted effort? Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology 122 (1) (1999) 37-44.

[42] R. Hjortebjerg, A. Flyvbjerg, J. Frystyk, Insulin growth factor binding proteins as therapeutic targets in type 2 diabetes. Expert Opinion on Therapeutic Targets 18 (2) (2014) 209-224.

[43] K. Kjaer-Sorensen, D.H. Engholm, M.R. Jepsen, M.G. Morch, K. Weyer, L.L. Hefting, L.L. Skov, L.S. Laursen, C. Oxvig, Papp-a2 modulates development of cranial cartilage and angiogenesis in zebrafish embryos. J. Cell. Sci. 127 (23) (2014) 5027-5037.

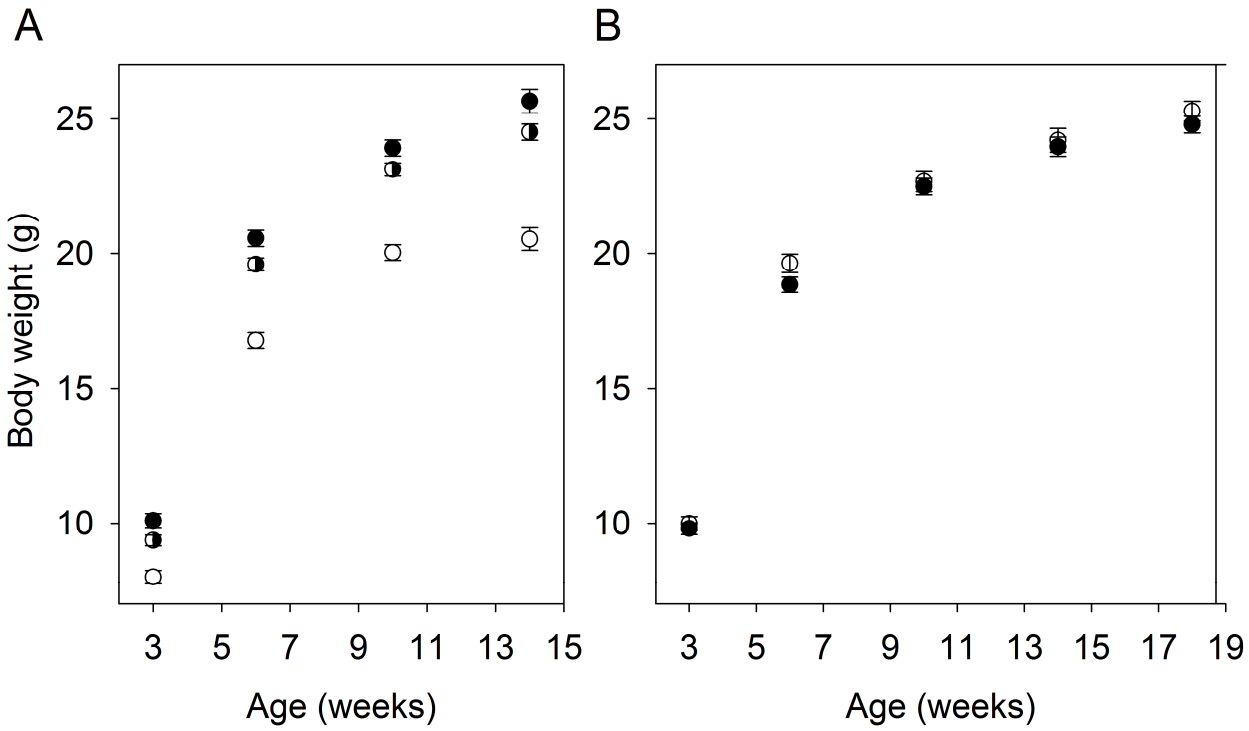


Figure 1. (A) Growth of mice homozygous for *Pappa2* disruption (*Pappa2*$^{KO/KO}$; open circles), heterozygous (*Pappa2*$^{fl/KO}$; half-filled circles), or homozygous for the floxed *Pappa2* allele (*Pappa2*$^{fl/fl}$; filled circles). Values are least squares means ± standard error from a general linear model including effects of genotype, sex and litter, i.e., males and females are pooled. (B) Growth prior to tamoxifen administration of mice carrying the tamoxifen-inducible *Cre-ERT2* transgene and homozygous for the floxed *Pappa2* allele (*Pappa2*$^{fl/fl}$; open circles) or homozygous for the wild-type allele (*Pappa2*$^{wt/wt}$; filled circles). Values are least squares means ± standard error from a general linear model including effects of *Pappa2* genotype, *Cre-ERT2* genotype, *Pappa2***Cre-ERT2* interaction, sex and litter, i.e., males and females are pooled. For clarity, littermates heterozygous for the *Pappa2* allele (*Pappa2*$^{fl/wt}$) or not carrying the *Cre-ERT2* transgene are not presented (see Supplemental Table 2).

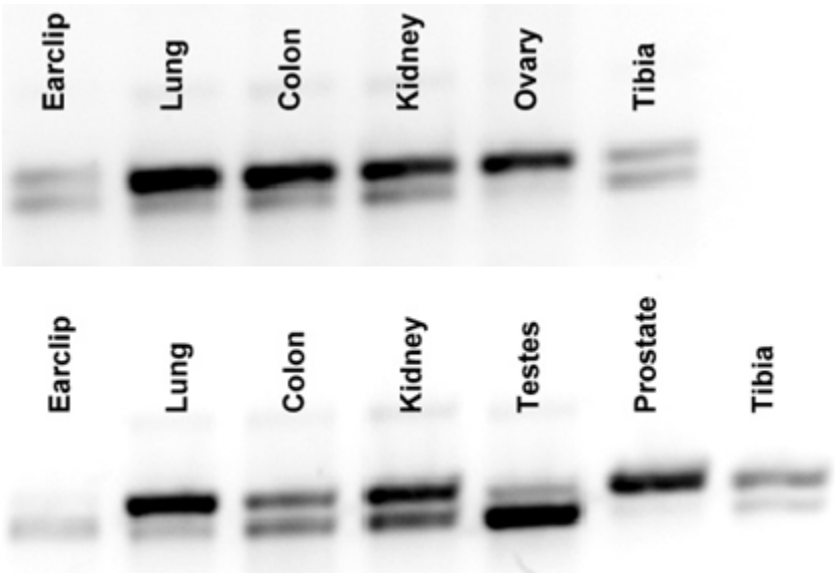
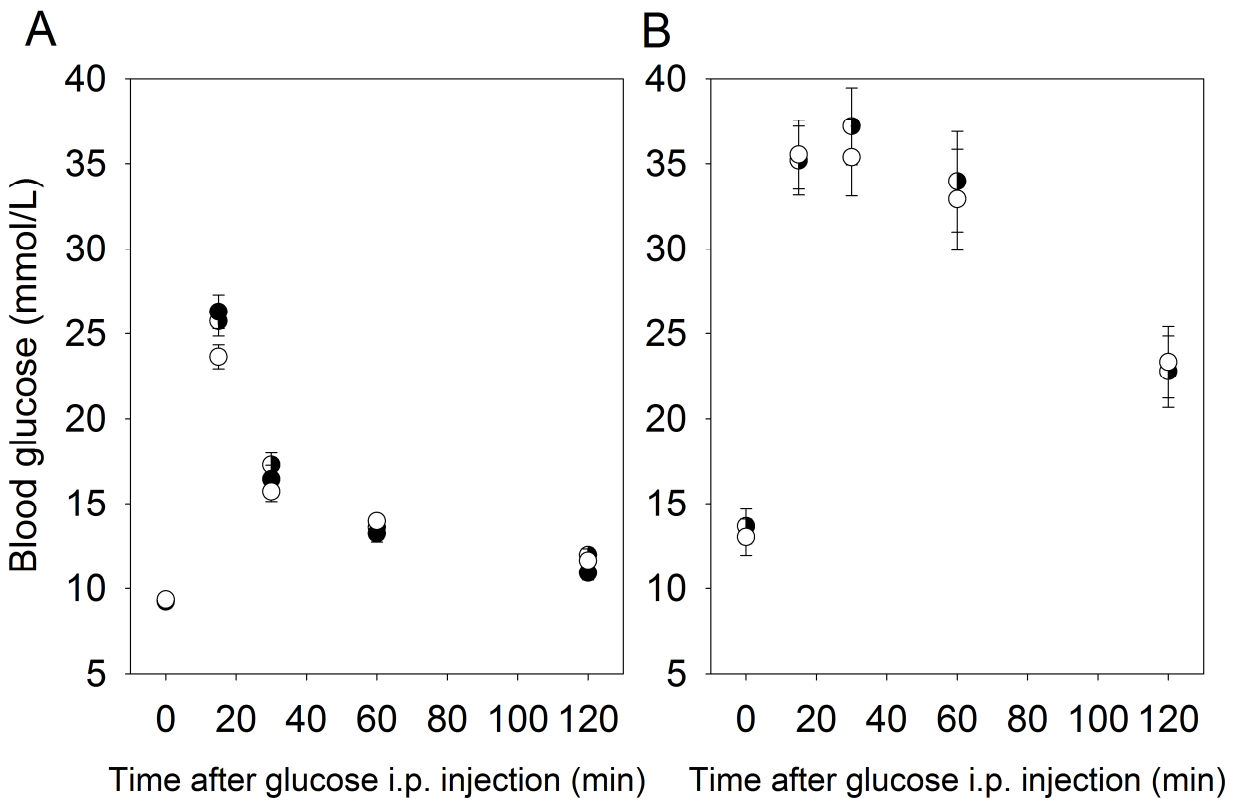


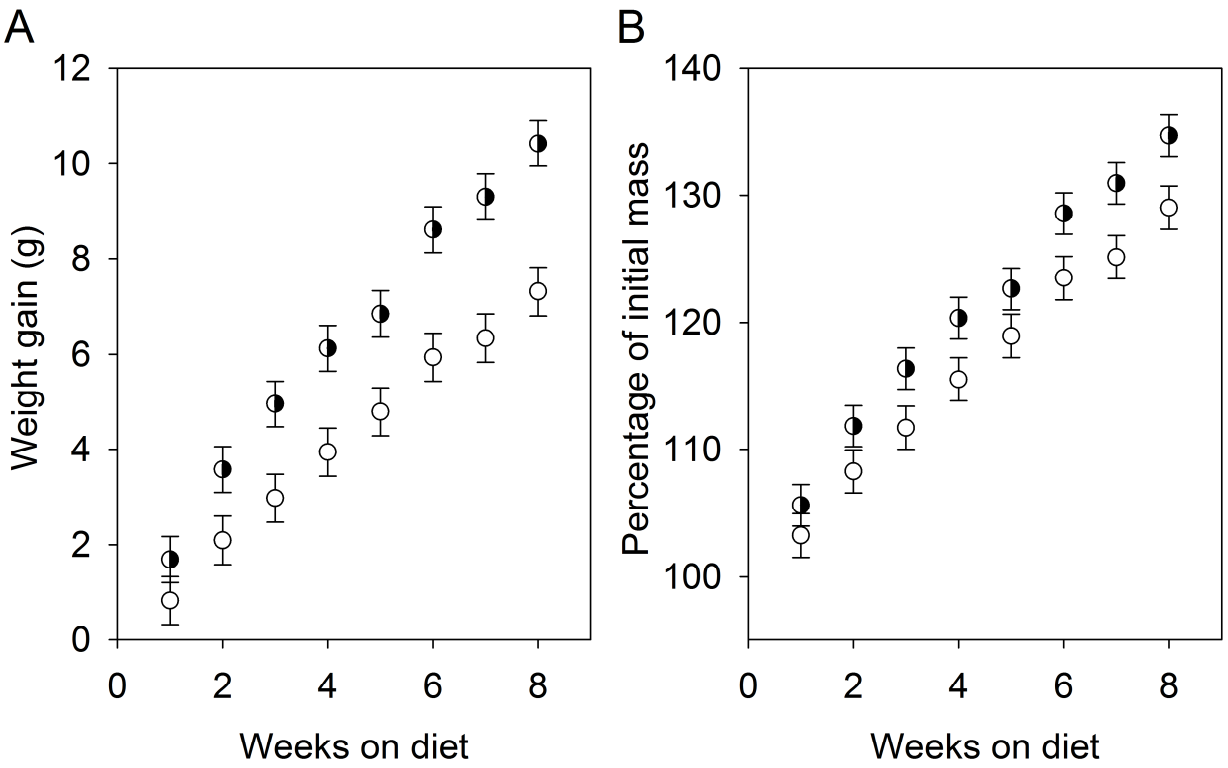
Figure 2. Partial disruption of the *Pappa2* gene in *Pappa2*$^{fl/fl}$; *Cre-ERT2* individuals collected 7 weeks after tamoxifen injection, as shown by PCR amplification of both the floxed (upper) and deletion (lower) *Pappa2* alleles in a female (upper) and male (lower).



548

549 Figure 3. Glucose tolerance test in (A) 11 week old males and females fed a chow diet and (B)
550 25 week old males fed a high-fat diet in mice homozygous for *Pappa2* disruption (*Pappa2*$^{KO/KO}$;
551 open circles), heterozygous (*Pappa2*$^{fl/KO}$; half-filled circles), or homozygous for the floxed
552 *Pappa2* allele (*Pappa2*$^{fl/fl}$; filled circles). Values are least squares means ± standard error from a
553 general linear model including effects of genotype, sex, and batch of testing (for mice on chow)
554 or genotype only (for mice on high-fat diet).

555



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557

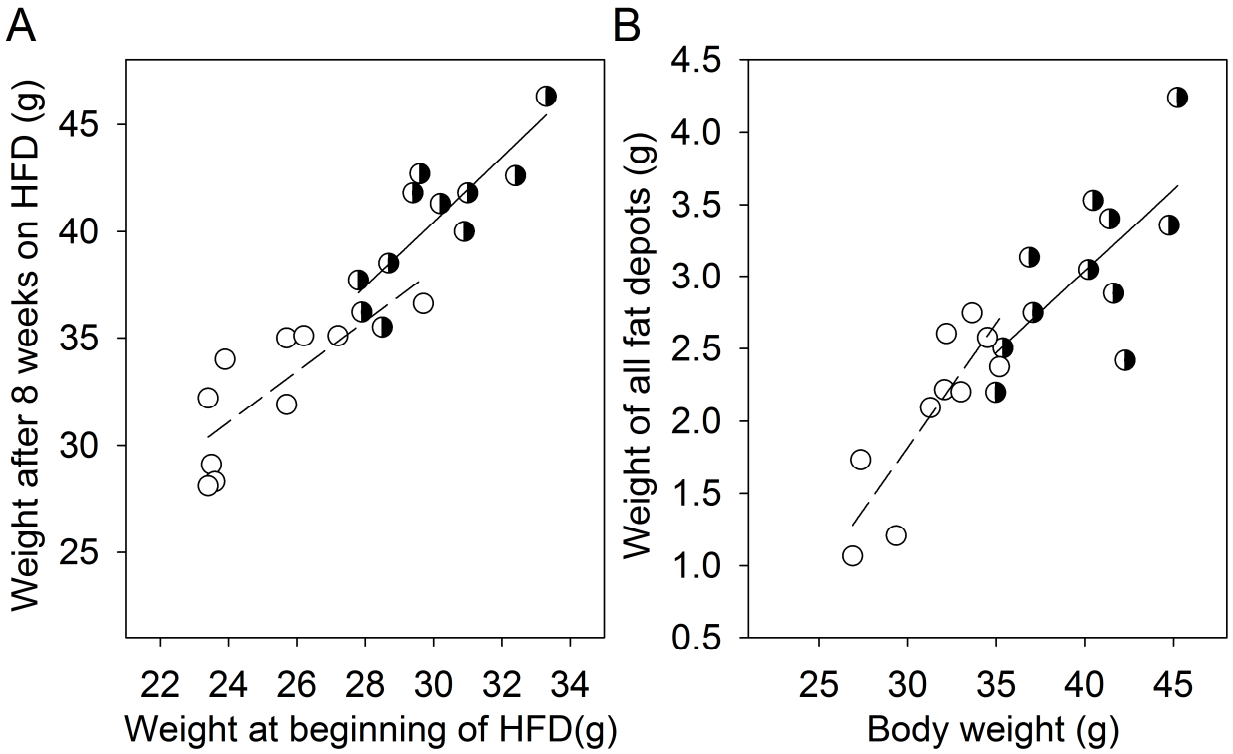
558 Figure 4. Effects of genotype on (A) weight gain (weight at the time indicated subtracted from

559 weight at the initiation of high-fat diet) and (B) body weight expressed as percentage of weight at

560 initiation of diet in mice homozygous for *Pappa2* disruption (*Pappa2*$^{KO/KO}$; open circles),

561 heterozygous (*Pappa2*$^{fl/KO}$; half-filled circles). Values are least squares means and error bars

562 indicate standard error.



563

564 Figure 5. Relationships between (A) weight after 8 weeks on high-fat diet (HFD) and initial body
565 weight and (B) total weight of fat depots after 8 weeks on high-fat diet and body weight in males
566 homozygous for *Pappa2* disruption (*Pappa2*$^{KO/KO}$; open symbols, dashed trendline) or
567 heterozygous for the floxed *Pappa2* allele (*Pappa2*$^{fl/KO}$; half-filled symbols, solid trendline).
568 While the trendlines shown were fitted separately for each genotype, the mass*genotype
569 interaction was not significant in any of these analyses ($P > 0.05$) and so was removed from the
570 model in analyses reported in the text.

571

572 Table 1. Components of the IGF pathway in mice homozygous for *Pappa2* disruption
573 (*Pappa2*$^{KO/KO}$), heterozygous (*Pappa2*$^{fl/KO}$), or homozygous for the floxed *Pappa2* allele
574 (*Pappa2*$^{fl/fl}$), from crosses between mice heterozygous for the *Pappa2* disruption.

	Pappa2$^{KO/KO}$	*Pappa2*$^{fl/KO}$	*Pappa2*$^{fl/fl}$	P-value[1]
Blood analytes measured at 6 weeks				
IGFBP-5 (ng/mL)	193 ± 4^{A}	126 ± 5^{B}	99 ± 4^{C}	0.0001
IGFBP-3 (ng/mL)	56 ± 24^{A}	604 ± 30^{B}	833 ± 27^{C}	0.0001
IGF-I (ng/mL)	945 ± 24^{A}	631 ± 31^{B}	581 ± 28^{B}	0.0001
Circulating and mRNA levels of IGFBPs at cull				
IGFBP-5 (ng/mL)	130 ± 7^{A}		90 ± 6^{B}	0.0011
Liver *Igfbp5* mRNA[2]	1.6 ± 0.4		2.6 ± 0.3	0.07
Kidney *Igfbp5* mRNA[2]	74.3 ± 12.5		87.3 ± 11.7	0.46
IGFBP-3 (ng/mL)	42 ± 10^{A}		260 ± 9^{B}	0.0001
Liver *Igfbp3* mRNA[2]	1.1 ± 0.5		1.9 ± 0.5	0.27
Kidney *Igfbp3* mRNA[2]	67.1 ± 11.4		49.9 ± 10.6	0.29

575 [1] The P-value is for the overall effect of genotype from a general linear model including effects
576 of genotype, sex and litter (blood analytes at 6 weeks) or genotype and sex only (traits at cull).
577 Values are least squares means ± standard error. Values with different superscripts are
578 significantly different using the Tukey-Kramer adjustment for multiple comparisons.
579 [2] mRNA units are fold-difference compared to a reference sample and corrected for β-actin.
580

581 Table 2. Body weight and circulating IGFBP-5 and IGFBP-3 levels in adult-specific *Pappa2*
582 deletion mice (*Pappa2*$^{fl/fl}$) and controls (*Pappa2*$^{wt/wt}$), all carrying the tamoxifen-inducible *Cre-*
583 *ERT2* transgene, 7 weeks after the first tamoxifen injection.

	Pappa2$^{fl/fl}$	*Pappa2*$^{wt/wt}$	P-value[1]
Weight (g)	27.49 ± 0.63	26.71 ± 0.54	0.39
IGFBP-5 (ng/mL)	157 ± 15	124 ± 13	0.12
IGFBP-3 (ng/mL)	258 ± 26	390 ± 23	0.0026

584 [1] The P-value is for the overall effect of genotype from a general linear model including effects
585 of genotype, sex and litter. Values are least squares means ± standard error.
586

Table 3. Baseline glucose and measures of glucose clearance in mice homozygous for *Pappa2* disruption (*Pappa2*$^{KO/KO}$), heterozygous (*Pappa2*$^{fl/KO}$), or homozygous for the floxed *Pappa2* allele (*Pappa2*$^{fl/fl}$), from crosses between mice heterozygous for the *Pappa2* disruption.

	Pappa2$^{KO/KO}$	*Pappa2*$^{fl/KO}$	*Pappa2*$^{fl/fl}$	P-value[1]
On chow diet				
Baseline blood glucose (mmol/L)	9.4 ± 0.2	9.3 ± 0.3	9.3 ± 0.3	0.92
Glucose tolerance test AUC (mmol*hour/L)	29.4 ± 0.5	30.3 ± 0.6	29.3 ± 0.7	0.47
Glucose tolerance test piAUC (mmol*hour/L)	10.6 ± 0.5	11.7 ± 0.6	10.8 ± 0.7	0.41
On high-fat diet				
Baseline blood glucose (mmol/L)	13.0 ± 1.1	13.7 ± 1.0		0.67
Baseline insulin (ng/mL)	3.4 ± 0.5	3.4 ± 0.5		0.99
Glucose tolerance test AUC (mmol*hour/L)	60.1 ± 4.4	61.3 ± 4.4		0.85
Glucose tolerance test piAUC (mmol*hour/L)	34.1 ± 3.1	33.5 ± 3.1		0.91

[1] The P-value is for the overall effect of genotype from a general linear model including effects of genotype, sex and batch (i.e., the day on which the glucose tolerance test was performed, to account for variation between test days) or genotype only (for traits measured on high-fat diet). Values are least squares means ± standard error.

Table 4. Fat depots from mice homozygous for *Pappa2* disruption (*Pappa2*$^{KO/KO}$) or heterozygous for the disrupted and floxed alleles (*Pappa2*$^{fl/KO}$) fed a high-fat diet.

Fat depot	*Pappa2*$^{KO/KO}$	*Pappa2*$^{fl/KO}$	P-value
Gonadal			
Percentage of body weight	4.1 ± 0.3	4.5 ± 0.3	0.19
Absolute weight (g)	1.3 ± 0.1	1.8 ± 0.1	0.002
Weight, controlling for body weight (g) [1]	1.5 ± 0.1	1.6 ± 0.1	0.58
Retroperitoneal			
Percentage of body weight	1.0 ± 0.1	1.4 ± 0.1	0.0004
Absolute weight (g)	0.31 ± 0.03	0.57 ± 0.03	0.0001
Weight, controlling for body weight (g) [1]	0.38 ± 0.04	0.50 ± 0.04	0.13
Omental			
Percentage of body weight	0.10 ± 0.03	0.15 ± 0.03	0.20
Absolute weight (g)	0.03 ± 0.01	0.06 ± 0.01	0.08
Weight, controlling for body weight (g) [1]	0.07 ± 0.01	0.03 ± 0.01	0.13
Mesenteric			
Percentage of body weight	1.4 ± 0.2	1.5 ± 0.2	0.79
Absolute weight (g)	0.45 ± 0.08	0.60 ± 0.08	0.21
Weight, controlling for body weight (g) [1]	0.73 ± 0.07	0.34 ± 0.07	0.005
Sum of fat depots			
Percentage of body weight	6.5 ± 0.4	7.6 ± 0.4	0.07
Absolute weight (g)	2.1 ± 0.2	3.0 ± 0.2	0.0013
Weight, controlling for body weight (g) [1]	2.7 ± 0.2	2.5 ± 0.2	0.54

[1] Values are least squares means ± standard error from a general linear model including genotype and body weight as a covariate.

599 Supplementary Table 1. Growth phenotypes in offspring homozygous for *Pappa2* disruption
600 (*Pappa2*$^{KO/KO}$), heterozygous (*Pappa2*$^{fl/KO}$), or homozygous for the floxed *Pappa2* allele
601 (*Pappa2*$^{fl/fl}$), from crosses between mice heterozygous for the *Pappa2* disruption.

	Pappa2$^{KO/KO}$	*Pappa2*$^{fl/KO}$	*Pappa2*$^{fl/fl}$	P-value[1]
Postnatal growth				
3 week weight (g)	8.00 ± 0.25^{A}	9.36 ± 0.20^{B}	10.10 ± 0.26^{B}	0.0001
3 week tail length (cm)	5.06 ± 0.04^{A}	5.50 ± 0.03^{B}	5.64 ± 0.04^{C}	0.0001
6 week weight (g)	16.77 ± 0.29^{A}	19.59 ± 0.24^{B}	20.56 ± 0.30^{C}	0.0001
6 week tail length (cm)	6.74 ± 0.04^{A}	7.36 ± 0.03^{B}	7.47 ± 0.04^{B}	0.0001
10 week weight (g)	20.03 ± 0.29^{A}	23.12 ± 0.24^{B}	23.92 ± 0.30^{B}	0.0001
10 week tail length (cm)	7.31 ± 0.03^{A}	8.00 ± 0.03^{B}	8.11 ± 0.03^{C}	0.0001
14 week weight (g)	20.53 ± 0.42^{A}	24.51 ± 0.30^{B}	25.66 ± 0.44^{B}	0.0001
14 week tail length (cm)	7.37 ± 0.04^{A}	8.21 ± 0.03^{B}	8.29 ± 0.05^{B}	0.0001

602 [1] The P-value is for the overall effect of genotype from a general linear model including effects
603 of genotype, sex and litter. Values are least squares means ± standard error. Values with
604 different superscripts are significantly different using the Tukey-Kramer adjustment for multiple
605 comparisons.

606

607 Supplementary Table 2. Growth phenotypes in offspring from matings between mice
608 heterozygous for the floxed allele and carrying the tamoxifen-inducible transgene (*Pappa2*$^{wt/fl}$;
609 *Cre-ERT2*) and mice heterozygous for the conditional allele (*Pappa2*$^{wt/fl}$). Values are least
610 squares means ± standard error from a general linear model including effects of *Pappa2*
611 genotype, *Cre-ERT2* genotype, *Pappa2*Cre-ERT2* interaction, sex and litter. *Pappa2* genotype,
612 *Cre-ERT2* genotype, *Pappa2*Cre-ERT2* interaction were not significant for any trait (P > 0.15 in
613 all cases).

	Cre-ERT2 transgene	*Pappa2*$^{fl/fl}$	*Pappa2*$^{wt/fl}$	*Pappa2*$^{wt/wt}$
3 week weight (g)	-	10.25 ± 0.26	9.86 ± 0.23	10.25 ± 0.21
	+	9.99 ± 0.26	10.08 ± 0.18	9.80 ± 0.22
3 week tail length (cm)	-	5.78 ± 0.06	5.80 ± 0.05	5.80 ± 0.04
	+	5.73 ± 0.05	5.78 ± 0.04	5.82 ± 0.05
6 week weight (g)	-	19.46 ± 0.34	19.08 ± 0.30	19.30 ± 0.28
	+	19.63 ± 0.34	19.45 ± 0.23	18.83 ± 0.28
6 week tail length (cm)	-	7.40 ± 0.06	7.41 ± 0.05	7.42 ± 0.05
	+	7.37 ± 0.06	7.48 ± 0.04	7.39 ± 0.05
10 week weight (g)	-	23.26 ± 0.41	22.41 ± 0.35	23.07 ± 0.30
	+	22.67 ± 0.37	22.57 ± 0.31	22.49 ± 0.31
10 week tail length (cm)	-	7.96 ± 0.07	7.95 ± 0.06	7.99 ± 0.05
	+	7.91 ± 0.06	8.01 ± 0.05	7.96 ± 0.05
14 week weight (g)	-	24.54 ± 0.48	24.00 ± 0.40	24.70 ± 0.36

	+	24.21 ± 0.44	24.32 ± 0.36	23.97 ± 0.36
14 week tail length (cm)	-	8.17 ± 0.07	8.16 ± 0.06	8.17 ± 0.05
	+	8.12 ± 0.06	8.24 ± 0.05	8.17 ± 0.05
18 week weight (g)	-	25.22 ± 0.41	24.92 ± 0.34	25.54 ± 0.30
	+	25.29 ± 0.37	25.43 ± 0.36	24.79 ± 0.31
18 week tail length (cm)	-	8.30 ± 0.07	8.28 ± 0.06	8.31 ± 0.05
	+	8.28 ± 0.07	8.37 ± 0.07	8.28 ± 0.06

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615